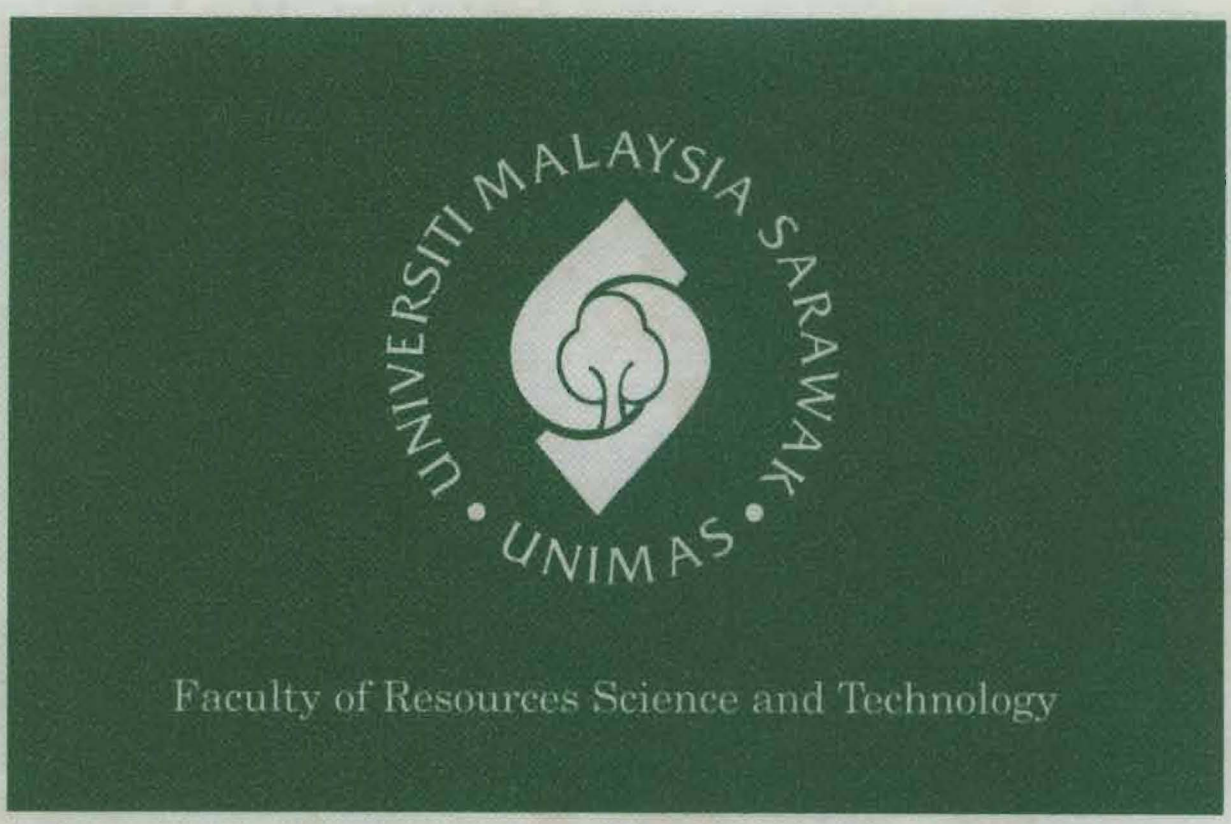

SCREENING FOR ANTIMICROBIAL ACTIVITIES IN MICROBES ISOLATED FROM MARINE BIOFILM

Liu Yu Choi

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Screening for Antimicrobial Activities in Microbes Isolated from Marine Biofilm

Liu Yu Choi (18890)

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Supervisor: Prof. Dr. Ismail bin Ahmad

Co-Supervisor: Dr. Siti Akmar Khadijah Ab Rahim

Resource Biotechnology

Molecular Biology

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

Declaration

I declare that this thesis entitled "Screening for Antimicrobial Activities in Microbes Isolated from Marine Biofilm" is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature : .....

Name : LIU YU CHOI

Date : 19 May 2010

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Contents

List of Abbreviations

CDA	Czapek-Dox Agar
HPLC	High Performance Liquid Chromatography
MDR	Multiple Drug Resistant
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimum Inhibitory Concentration
MR	Methyl-red
MRSA	Methicillins-resistant *Staphylococcus aureus*
NA	Nutrient Agar
PDA	Potato Dextrose Broth
PVC	Polyvinyl chloride
SIM	Hydrogen sulphite, H_2S-Indole-Motility
VP	Voges-Proskauer
VREF	Vancomycin-resistant *Enterococcus faecium*

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Liu Yu Choi

Resource Biotechnology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

The emergence of multi-drug resistant (MDR) bacteria has lead to an increase demand for new antibiotics. Microbes isolated from marine biofilms offer a potential source of novel antibiotics due to their unique living circumstances in marine environment. A study of antibacterial activity in microbes isolated from marine biofilms found on the surface of marine samples collected from Satang Island, Sarawak was carried out. A total of 108 bacteria and 25 fungi were isolated. From that, 76 bacterial isolates and 20 fungal isolates were subjected to preliminary screening using overlay-agar technique against the Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) test bacteria. Six bacterial isolates were found active against the test bacteria while 15 out of 20 fungal isolates showed activities against the test bacteria. The six putative antibacterial isolates were subjected to secondary screening using agar well diffusion method where isolate P5.1.2 showed the most potential of producing antibacterial substances with the largest inhibition zone against all the test bacteria in both preliminary and secondary screening and therefore selected for further study. Seven fungal isolates (4.1.1, 4.1.2, P2.2.1, P8.1.2, P10.2.1, P1.2.1 and P8.1.1) which showed the strongest antibacterial activities were selected for further study based on the results of the preliminary screening. A total of eight isolates were subjected to the antibiotic production on solid media and subsequent extraction using methanol. These isolates were identified up to the genus level. Bacterial isolate P5.1.2 was putatively identified as *Klebsiella* sp. after five biochemical tests were performed. Six out of seven fungal isolates were successfully identified using slide-culture method. Isolates P8.1.2, P10.2.1 and P8.1.1 were putatively identified as *Penicillium* sp. while isolates 4.1.2, P2.2.1 and P1.2.1 were putatively identified as *Bipolaris* sp., *Endophragmia* sp. and *Drepanoconis* sp., respectively. Isolate 4.1.1 remain unidentified due to the lack of spore formation. Methanol extracts from selected bacterial and fungal isolates were subjected to antibacterial screening against four test bacteria (*Staphylococcus aureus, Escherichia coli, Salmonella typhi and Enterobacter aerogenes*) using agar well diffusion method. Six out of eight extracts showed antibacterial activities. These results suggest that microbes isolated from marine biofilms have great potential for antibiotic production. Further studies are required to determine the most suitable antibiotic production medium to be used to increase the concentration of antibiotics obtained.

Keywords: Antibacterial activity, marine microbes, biofilms

ABSTRAK

Kemunculan bakteria rintang ubat-berganda (MDR) telah meningkatkan permintaan terhadap antibiotik baru. Mikrob yang dipencilkan daripada bio-filem laut berpotensi sebagai sumber antibiotik baru disebabkan oleh keunikan cara hidupnya di dalam laut. Satu kajian terhadap aktiviti antibakteria untuk mikroorganisma yang dipencilkan daripada bio-filem yang terdapat pada permukaan sampel laut yang diperoleh daripada Pulau Satang, Sarawak telah dijalankan. Sejumlah 108 pencilan bakteria dan 25 pencilan kulat diperoleh. Daripada jumlah tersebut, penyaringan pertama telah dilakukan terhadap 76 pencilan bakteria dan 20 kulat terhadap bakteria ujian Gram-positif (Staphylococcus aureus) dan Gram-negatif (Escherichia coli) dengan teknik agar-overlay. Enam daripada 72 pencilan bakteria dan 15 daripada 20 pencilan kulat didapati aktif terhadap bakteria ujian. Penyaringan sekunder dilakukan terhadap pencilan bakteria yang aktif tersebut dengan menggunakan kaedah resapan telaga agar dimana pencilan P5.1.2 menunjukkan potensi penghasilan bahan antibakteria. yang paling tinggi dengan zon inhibisi yang paling besar untuk kedua-dua penyaringan. Tujuh pencilan kulat (4.1.1, 4.1.2, P2.2.1, P8.1.2, P10.2.1, P1.2.1 and P8.1.1) yang menunjukkan aktiviti antibakteria yang kuat dipilih untuk kajian yang seterusnya berdasarkan keputusan penyaringan pertama. Sejumlah lapan pencilan telah dipilih untuk penghasilan antibiotik menggunakan media pepejal dimana pengekstrakan dilakukan dengan menggunakan metanol. Kesemua pencilan tersebut dikenalpastikan kepada tahap genus. Pencilan bakteria P5.1.2 telah dikenalpastikan sebagai Klebsiella sp. selepas lima ujian biokimia dijalankan. Enam daripada tujuh pencilan kulat dapat dikenalpastikan dengan kaedah kultur-slid. Tiga pencilan kulat iaitu P8.1.2, P10.2.1 dan P8.1.1 dikenalpastikan sebagai Penicillium sp. manakala pencilan 4.1.2, P2.2.1 dan P1.2.1 masing-masing dikenalpastikan sebagai Bipolaris sp., Endophragmia sp. dan Drepanoconis sp. Pencilan 4.1.1 tidak dapat dikenalpastikan kerana ketiadaan pembentukan spora. Penyaringan antibakteria untuk ekstrak metanol daripada pencilan terpilih telah dilakukan terhadap empat bakteria ujian (Staphylococcus aureus, Escherichia coli, Salmonella typhi dan Enterobacter aerogenes) dengan menggunakan kaedah resapan telaga agar. Enam daripada lapan ekstrak menunjukkan aktiviti antibakteria. Keputusan ini menunjukkan mikrob yang dipencilkan daripada bio-filem laut berpotensi untuk penghasilan antibiotik. Kajian lanjut adalah diperlukan untuk menentukan medium penghasilan antibiotik yang paling sesuai untuk digunakan supaya kepekatan antibiotik yang lebih tiggi dapat diperoleh.

Kata kunci: Aktiviti antibakteria, mikroorganisma laut, bio-filem

1.0 Introduction

An antibiotic or antimicrobial agent is a chemical substance that is produced by microorganism either by a bacterium or a fungus having the capacity to kill or inhibit the growth of other microorganisms in dilute solution (Vandamine, 1984). According to Demain and Sanchez (2009), antibiotics play an important role in treating bacterial infections and are widely used for medical purposes. However, the overuse of antibiotics may lead to the emergence or re-emergence and the spread of antibiotic-resistant bacteria or even the multiple-drug resistant (MDR) bacteria (Miranda and Zemelman, 2001). Therefore, the screening for new antibacterial substances is vital in order to cope with the emergence of antibiotic-resistant bacteria and the emergence of new diseases.

In the past, most of the antibiotic-producing microbes have been discovered and isolated from terrestrial environment but little work has been done on marine environment due to sampling difficulties (Faulkner, 2000). Although marine microorganisms are not well defined taxonomically, preliminary studies had indicated the wealth of microbial diversity in the sea, making it a promising frontier for the discovery of new drugs (Blunt *et al.*, 2004). Previous studies had shown that marine microbes were great source for antibiotic screening as there were many new antibiotics had been discovered including pelagiomicins from *Pelagiobacter variabilis* (Imamura, 1997), pyrones from *Pseudomonas* (Singh *et al.*, 2003) and a new antibiotic, thiomarinol from a marine bacterium *Alteromonas rava* (Shiozawa *et al.*, 1993).

Bacteria or any other microorganisms that live on the surface of marine organism are subjected to a highly competitive environment due to limited space and nutrients. Such bacteria have been documented to possess higher antimicrobial activities than those free-living bacteria in marine environment (Lemos *et al.*, 1985). Since there is a great diversity

of microbes colonizing all surface in the marine environment, screening for antimicrobial activities in such microbes is therefore essential for the discovery of new antibiotics.

Marine biofilm is a thin layer of film made up of biomolecules, bacteria, fungi, protozoa and diatoms that cover both the animate and inanimate surfaces submerged in seawater (Clare *et al.*, 1992). Marine biofilms are believed to have the potential for the isolation of marine microbes with antimicrobial properties (Armstrong *et al.*, 2001). Hence, a study was proposed to isolate such microbes and was maximized with the addition of sea water to the isolation media. Both bacterial and fungal isolates were screened for their antibacterial activities against the Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) test bacteria.

Bacterial isolates were subjected to preliminary screening and subsequent screening by spot inoculation and agar-well diffusion method, respectively. Fungal isolates were screened twice for the presence of antibacterial activities using the same overlay-agar technique but grown on different growth media. This allowed the selection of isolates with most potential of producing antibiotics for the extraction of antibiotics using methanol solvent. Identification and characterization of these selected isolates was performed. Finally, agar well diffusion assay was carried out to determine the relative inhibitory strength of extracted antibiotics.

The objectives of this study are:

1. To isolate marine microbes from the biofilms of marine samples.
2. To screen for the presence of antibacterial activities in isolated microbes.
3. To identify and characterize isolates with antibacterial activities.
4. To extract the antibiotics from isolates with most potential of producing antibiotics.
5. To determine the relative inhibitory strength of extracted antibiotics.

2.0 Literature Review

2.1 Antimicrobial Compounds

The term of 'antibiotic' and 'antimicrobial' may be used effectively and interchangeably irrespective of the specific source of the chemical entity. This substance can be further subdivided predominantly into two categories, namely antibacterial and antifungal depending on the organism that is inhibited (Sambamurthy and Kar, 2006). According to Kayser *et al.* (2005), antimicrobial agents are categorized into broad, narrow, or medium spectrum of action and the efficacy, or effectiveness of an antimicrobial substance refers to its bactericidal or bacteriostatic effect.

According to Hogg (2005), all antibiotics have common mechanisms of action in their interference with the normal function of the target cell. Most of the antibiotics exert their effect by inhibition of cell wall synthesis, disruption of cell membranes, interference with protein synthesis or interference with nucleic acid synthesis. Determination of the mechanism of action is necessary before an antibiotic can be subjected to an *in vivo* testing (Kayser *et al.*, 2005).

2.2 Antibiotic-resistant Bacteria

The widespread use of antibiotics for treating human infection has lead to the emergence and the spread of multiple-drug-resistant (MDR) bacteria in various environments (Livermore, 2000). According to Demain and Sanchez (2009), bacteria have existed on Earth for several billions years in which they have been exposed to a wide range of naturally occurring antibiotics. Developing the antibiotic-resistant mechanism is their only way to survive. Therefore, it is not surprising that the bacteria have resistant to most of the natural antimicrobial agents that have been developed over the past 50 years.

In addition, antibiotic resistance will greatly reduce the effectiveness of antimicrobial drugs that are currently available against pathogenic bacteria such as methicillins-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VREF) where these bacteria are not susceptible to most of the conventional antibiotics (Desbois *et al.*, 2008). The emergence of multi-drug resistant (MDR) bacteria is causing an increased concern in healthcare institutions worldwide, where discovery of novel antibacterial compounds for biomedical exploitation is being pursued in order to combat these problematic bacteria (Sugie *et al.*, 2002). Although many synthetic antibiotics have been developed using pre-existing antibiotics to combat the multi-drug resistant bacteria, new class of antibiotic is needed to overwhelm the mutation rate of known resistant mechanism in these bacteria, particularly the MRSA and VREF (Burges *et al.*, 1999).

2.3 Marine Environment

Marine environment is an immense and practically unexploited source of novel and potentially useful bioactive compounds such as antibacterial, antifungal and antiviral substances (Ninawe, 2006). Recently, marine microorganisms have been recognized as a potential source of novel secondary metabolites due to redundancy and lack of novelty in the extract of soil-derived bioactive compounds (Toledo *et al.*, 2006). According to Ninawe (2006), most of the marine microbes possess of unique metabolic, physiological properties and functions that will not only ensure their survival in extreme marine environment but also offer a tremendous source of novel enzymes and bioactive metabolites for potential exploitation.

In addition, taxonomically diverse bacterial species have been found within the marine environment. These bacteria exhibit unique structural and physiological

characteristics that enable them to survive extreme pressure, salinity and temperature with potential production of novel secondary metabolites and bioactive compounds that is different from terrestrial microbes (Eccleston *et al.*, 2008). According to Abraham (2004), oceans are a promising source of novel pharmacologically active compounds due to the fact that the oceans cover more than 70 percent of the earth's surface. Besides, the natural antimicrobial activities of seawater are believed to be closely associated with marine bacteria which produce antimicrobial substances (Rosenfeld and Zobell, 1947).

2.4 Marine Biofilm

All submerged surfaces in the marine environment are constantly being exposed and colonized by living organisms ranging from microscopic organisms such as bacteria to macro-invertebrates (Lee and Qian, 2004). This phenomenon is known as epibiosis when the colonization occurs on animate surfaces. Every surface that is immersed in the sea will be coated with biofilms rapidly including those of marine organisms (Armstrong *et al.*, 2001). Animate surface provides a habitat that is rich in organic material either as a result of the physical process in which molecules adsorb to the surface or due to the production of mucus or mucilage by the organism.

Marine invertebrates such as sea cucumber and sea urchin are believed to have the potential for the isolation of microbes with antimicrobial activities due to competition among the microbes for limited space and nutrients on living surface will encourage these microbes to produce antibiotics (Zheng *et al.*, 2005). According to Jangoux (1987), Echinoderms offer a great source of substratum for attachment or settlement and the growth of bacteria as they often occur in high abundance and therefore a potential source for the isolation of antibiotic-producing marine microbes.

2.5 Antibiotic Producing Microbes from Marine Biofilm

Competition for space and nutrients exert a selective pressure among marine microbes which induce these microbes to produce many natural products that may possess medical or industrial values (Armstrong *et al.*, 2001). In a study by Cueto *et al.* (2001), a new chlorinated benzophenone compound, pestalone which showed antibacterial activity against MRSA (MIC=37 ng/ml) and VREF (MIC=78 ng/ml) was found to be produced by a marine fungus, *Pestalotia* sp. that was isolated from the surface of the brown algae *Rosenvingea* sp.

Besides, marine epiphytic bacteria that are associated with nutrient rich algal surface and invertebrates have also been shown to produce antibacterial secondary metabolites, which inhibit the settlement of potential competitors (Bernan *et al.*, 1997). In a study by Abraham (2004), marine pigmented bacterium *Alteromonas* sp. isolated from *Penaeus monodon* Fabricius larva were shown to exhibit inhibitory activity against pathogenic strain of *Vibrio* sp. Hence, marine microbes that colonized the surface of marine organism offer a potential source for the discovery of new antimicrobial drugs.

2.6 Antimicrobial Screening Assays

2.6.1 Spot Inoculation and Overlay-agar Technique

Spot inoculation is a method that is frequently used in combination with overlay-agar technique for the screening of antimicrobial activities in microbial isolates (Dharmaraj and Sumantha, 2009). The assay plates consist of two layers with lower basal agar layer seeded with bacterial or fungal isolates and being overlaid with another layer of 0.75% soft agar seeded with test bacteria on top of the basal agar (Omura, 1992). The resulting inhibition zones formed around the colonies is due to the presence of antibacterial activities.

2.6.2 Agar-diffusion Assay

Agar diffusion refers to the movement of molecules through the matrix that is formed by the gelling of agar (Narins, 2003). It is used to determine the susceptibility or resistance of a bacterial strain to an antibacterial agent based on the principle that the degree of molecules movement through the gelling agar can be related to the concentration of the molecules. The most common method in agar-diffusion based antibiotic sensitivity testing is the agar well diffusion method where agar plagues were removed, resulting in a number of wells which can be filled with antibiotics and screened for the presence of antibacterial activities against the test bacteria seeded on the agar surface (Rahman *et al.*, 2005).

After an overnight incubation, the diameter of a clearing zone around the well is measured and compared with the zones of inhibition produced by solutions of known concentrations of standard antibiotics. The presence of suspended particles in the test samples or precipitation of water-insoluble substances in the wells could interfere with the diffusion of the antimicrobial substance where warming the plate is necessary to accelerate the diffusion of antimicrobial substance into the media (Rahman *et al.*, 2005).

The resulting circular zone of inhibition is due to the diffusion of antibiotic through the agar well which eventually inhibits the growth of the test bacteria that are seeded on the plate (Rahman *et al.*, 2005). According to Vandepitte *et al.* (2003), the test must be standardized since the zone size will be affected by the size of inoculums, medium compositions, incubation temperature, excess moisture and the thickness of the agar. If these conditions are uniform, the difference in diameter of zone will be mainly due to the difference in the strength of antibiotics being tested.

3.0 Materials and Methods

3.1 Sampling

Two batches of marine samples were brought to the laboratory on 2nd of August and 4th of October 2009 for the isolation of marine microbes from the biofilms that were present on the surface of the samples. These samples were collected at Satang Island, Sarawak by the crew from Aquatic Science Department. Polyvinyl chloride or PVC plates as described by Bourque *et al.* (2007) were placed in the bucket tied with weights and then submerged into the sea for approximately two days to allow the formation of biofilms on it. Living samples such as sea cucumber and sea urchins were collected and kept alive in containers filled with seawater prior to the isolation procedure.

3.2 Isolation and Cultivation of Marine Microbes

3.2.1 Preparation of Culture Media

Potato Dextrose Agar (PDA) and Nutrient Agar (NA) were prepared with the addition of 10% natural seawater that has been autoclaved for the isolation and cultivation of marine microorganisms. Autoclaved seawater and distilled water were mixed in a proportion of 1:9 before it was added to the powdered media. The media were boiled using a stirring hotplate and then autoclaved at 121°C, 15 psi for 20 minutes. After that, the media were cooled down in water bath at 50°C and then poured onto the Petri dishes in the laminar hood to prevent contamination (Benson, 2001). The plates were stored at 4°C after the media have solidified.

3.2.2 Isolation of Microbes from Marine Biofilm

The surface of sea cucumber and the PVC plates were rinsed with the autoclaved sea water to remove gross contaminants and sediments. The surface of the samples were swabbed

using a sterile cotton swab and then swabbed directly onto two agar plates, potato dextrose agar (PDA) and nutrient agar (NA) respectively, for the isolation of microbes from marine biofilms. This procedure was performed under sterile environment (Benson, 2001). All the plates were then incubated at room temperature (28°C). NA plates were incubated for 1 to 2 days whereas PDA plates were incubated for 3 to 4 days.

3.2.3 Cultivation and Storage of Microbial Isolates

Isolated bacterial and fungal colonies were cultivated on a fresh plate using the same culture media from which it was derived. Bacteria were incubated for 1 to 2 days whereas fungi were incubated for 4 to 7 days, both at room temperature (28°C). Bacterial isolates were sub-cultured at least twice using the streak plate method as described by Prescott *et al.* (2002). Fungal isolates were sub-cultured by transferring a small piece of its mycelial growth onto the surface of new agar plates where the mycelial growth was in contact with the agar. After pure isolates were obtained, both bacterial and fungal isolates were inoculated onto slant agars and incubated at similar incubation temperature and length. The slant agars as described by Green (2009) were stored at 4°C as a stock culture after incubation.

3.3 Antibacterial Screening of Bacterial Isolates

3.3.1 Preliminary Screening: Spot Inoculation Method

Isolated marine microbes were screened for the presence of antibacterial activities using the overlay-agar technique as described by Fleming *et al.* (1975). Bacterial isolates were sub-cultured from its stock culture onto PDA or NA depends on which it derived and incubated at 28°C for 2 days. Bacterial isolates were seeded on the agar plates using spot inoculation method with maximum of 12 bacterial spot per agar plate and then incubated at

28°C for 3 days before being overlaid with soft agar as described by Oskay (2009). Each isolate was replicated for the screening against each test bacteria.

Preliminary screening was performed against two test bacteria, Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*). The test bacteria were prepared on NA and incubated at 37°C for overnight. Single colonies were then inoculated into nutrient broth (NB) and incubated at the same condition. The final concentration of the test bacterial suspension was adjusted to OD = 0.6 at 520nm before 100µl of the test bacterial suspension were added to the soft agar (NA) (Dalal *et al.*, 2009). The soft NA media were then overlaid onto the agar plates that were seeded with the bacterial isolates and then incubated at 28°C for 24 hours. After incubation, the plates were checked for the presence of inhibition zone around the bacterial spots as a result of antibacterial activities.

3.3.2 Secondary Screening: Agar-well Diffusion

Bacterial isolates that showed antibacterial activities in the preliminary screening were selected for secondary screening using the agar-well diffusion method as described by Valgas *et al.* (2007). Test bacteria were prepared and standardized as in preliminary screening. The test bacterial inoculums were spread uniformly over the surface of NA plates. Six wells of 4.0mm diameter each were made in the plates with a sterile cork borer. The wells were then filled with 50µl of bacterial suspension of each bacterial isolates that were incubated at room temperature (28°C) for two days in NB. The inoculated plates were incubated at 37°C for 1 hour without the plate being inverted to allow the pre-diffusion of bacterial suspension into the agar before the plate were inverted and incubated for 24 hours. The plates were examined for the presence of inhibition zones which appeared as a clear area around the wells.

3.4 Antibacterial Screening of Fungal Isolates

3.4.1 Antibacterial Screening of Isolates grown on PDA or NA

Fungal isolates were sub-cultured from slant agars onto PDA or NA, depending on which growth media they were originally isolated from. After 5 days incubation at 28°C, the fungal isolates were sub-cultured to new plates with maximum of four isolates per plate and incubated at 28°C for 2 days before screened against the test bacteria using same overlay-agar technique (Fleming *et al.*, 1975). Fungal isolates were screened for the presence of antibacterial activities against the same test bacteria which were prepared in the same way as in the preliminary screening for bacteria. After incubation, the plates were checked for the presence of inhibition zone around the fungal colonies as a result of antibacterial activities.

3.4.2 Antibacterial Screening of Isolates grown on CDA

Fungal isolates that showed antibacterial activities in the preliminary screening were sub-cultured from the stock culture onto PDA or NA and incubated at 28°C for 5 days. After 5 days of incubation, the fungal cultures were transferred using a sterile cork borer onto CDA that were also prepared with the addition of 10% sea water and then incubated at 28°C for 3 days before overlaid with soft agar seeded with test bacteria. After 24 hours of incubation at 28°C, the plates were checked for the presence of inhibition zone around the fungal colonies as a result of antibacterial activities.

3.5. Bacteria Identification

Gram-staining as described by Benson (2001) was carried out on the bacterial isolate which showed the most potential of producing antibiotic through the antibacterial screening. A total of five standard biochemical tests were performed based on the Gram-

staining result to identify the isolate: oxidase test, SIM test (Hydrogen-Sulfide, Indole and Motility), methyl-red (MR) test, Voges-Proskauer (VP) test and citrate utilization (Harley & Prescott, 2002). Isolates which showed the strongest antibacterial activity were selected to be identified up to genus level based on the Gram-stain and biochemical properties with the aid of Bergey's Manual of Determinative Bacteriology (Bergey *et al.*, 1994).

3.6 Identification and Characterization of Fungi

Fungal isolates which showed the most potential of producing antibiotics in the antibacterial screening were selected for identification and characterization.

3.6.1 Macroscopic Examination

The growth characteristics of selected fungal isolates were observed after the fungal colony has grown to full plate. Observation were made on the colour of the mycelia mat, reverse colour, margin of the colony (smooth, irregular or looped), mycelia mat characteristics (flat, raised, cottony or powdery), and the colour change of culture media (Maza *et al.*, 1997).

3.6.2 Microscopic Examination: Slide-Culture Method

The identification of fungi was carried out through microscopic examination using slide-culture method (Maza *et al.*, 1997). A small sample consisting of agar and fungal growth was cut out from a fungal culture, and was aseptically transferred onto a sterilised microscope slide. The sample was then covered with a cover slip, which was supported by plasticins. The culture slides were placed in Petri dish and sealed with parafilm and then incubated at 28°C for 2 to 3 days. When the fungi matured, the slides were examined under the microscope for the spores, spore structure and the hyphae structure (septate or aseptate and dichotomous or non-dichotomous branching). The identification of fungi was based on

The Saccardo System of Classification with the aid of descriptions found in Illustrated Genera of Imperfect Fungi (Barnett and Hunter, 1972).

3.7 Extraction of Antibiotic

Selected microbial isolates were grown on solid agar until the colonies had reached maturation. The agar plates were dried at room temperature to remove most of the water that is present in the agar. Dried agar were peeled off from Petri dish and cut into small pieces before being submerged under 100% methanol of known volume to dissolve the antibiotic (Bie *et al.*, 2005). Dissolved antibiotic were left at room temperature to evaporate the solvent until a small volume of concentrated liquid was obtained. The final volume of the liquid was measured and kept under 4°C in an Eppendorf tube.

3.8 Relative Inhibitory Strength of Extracted Antibiotics

3.8.1 Test Bacteria Preparation

Gram-positive bacteria, *Staphylococcus aureus,* and Gram-negative bacteria, consisting *Escherichia coli, Salmonella typhi,* and *Enterobacter aerogenes*, were chosen to be tested for their sensitivity against the extracted antibiotics. The test bacteria were prepared on Mueller-Hinton agar (MHA) and incubated at 37°C for overnight. Single colonies were then inoculated into Mueller-Hinton broth (MHB) and incubated at similar condition. The final concentration of the test bacterial suspension was adjusted to OD = 0.168 at 550nm (Ahmed *et al.*, 2008).

3.8.2 Initial Standard Determination

One of the liquid left during the extraction process was subjected to serial dilution using sterile distil water. The test bacterial inoculums were spread uniformly over the surface of NA plates. Six wells of 4.0mm in diameter each were made using sterile cork borer and